The Anticancer Mechanism of an Approved Disease Modifying Herb Medicine: Total Glucosides of Paeony Target both EGFR and HER-2 in Lung Cancer Cell Lines

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Abstract

The high cost and slow pace of the pharmaceutical industry in bringing novel anti-cancer drugs for waiting patients indicates that the current business model for drug development needs innovation. We assumed that studying anticancer activities from approved disease modifying medicines might provide a possible way to lower the cost and speed up anti-cancer drug development. To test the idea, we studied the molecular mechanisms of anticancer effect of Total Glucosides of Paeony (TGP), a Chinese State Food and Drug Administration approved drug, in non-small cell lung cancer (NSCLC) cell-based systems. NSCLC is the leading cause of death from all cancers.

Introduction

Despite exponentially increased investment, the total numbers of approved drugs by US FDA have been about the same each year during the past 50 years [1]. Market capitalization of 9 top-tier pharmaceutical companies from 2001 to 2009 had accumulative loss of $626 billion [1].

Therefore, identifying and successfully developing new drug candidates while trying to contain the costs might be the key to make pharmaceutical industry profitable and novel drugs available for waiting patients. New ideas, new sources, and new data might help the pharmaceutical industry to speed up the process of identifying new drug candidates. Based on this idea, we studied the molecular mechanisms of anti-cancer effect of Total Glucosides of Paeony (TGP), a Chinese State Food and Drug Administration approved rheumatoid arthritis modifying drug, in a non-small cell lung cancer (NSCLC) cell-based system.

Non-small-cell lung cancer (NSCLC) is the leading cause of death from all cancers [2,3]. Epidermal growth factor receptor (EGFR) is a major and effective molecular target of anti-NSCLC therapy because EGFR is over-expressed in 50–80 percent of NSCLC [4].

Autophosphorylation of tyrosine residue of EGFR by its tyrosine kinase domain initiates activation of the downstream signaling cascades PI3K–Akt, which has anti-apoptotic and pro-survival effects that promote cancer cell growth [5]. Tyrosine kinase inhibitors (TKIs) directed against EGFR, such as gefitinib or erlotinib, are among the first molecular-targeted agents to be approved in the US and other countries for the treatment of NSCLC, breast, head and neck cancers [6], esophagus cancer [7], glioblastoma [8], and epithelial ovarian cancer [9]. However, cancer patients who have benefited from costly EGFR TKI therapy quickly generate resistance to the drug with 70% known [10-12] and 30% unknown reasons [4]. Hence there is a great need to develop cheaper and more effective TKIs.

Paeonia radix, the dried root of Paeonia lactiflora Pall, has been prescribed in various formulations of Chinese medicines for over 1500 years [13]. Modern research indicates that the total glucosides of paeony (TGP), an active fraction extracted from Paeonia radix, is responsible for many of its therapeutic properties. The major components (Figure 1A) of TGP include paeoniflorin (~90%), oxypaeoniflorin, and benzoylpaeoniflorin, which all have the same florin core structure [14-16].

Keywords: Total glucosides of paeony; Lung cancer; Cell cycle arrest; Apoptosis; EGFR; HER-2

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Figure 1: (A) The major components of the total glucosides of paeony (TGP). (B, C and D) Growth inhibitory effect of TGP and positive control (Gefitinib) on A549 and H1299 human lung cancer cells. A549 (B), H1299 (C) cells were seeded in 96-well plates, and after 24 h, cells were treated with serial concentrations of TGP and Gefitinib (D) for 8 h, 32 h and 56 h of treatments, followed by adding resazurin to the media for 16 h. The fluorescent signal is monitored using 544 nm excitation wavelength and 595 nm emission wavelength by Spectramax M5 plate reader (Molecular Devices). The relative fluorescence unit (RFU) generated from the assay is proportional to the number of living cells in each well. Data are mean ±SD (n=6).

TGP has shown to be anti-inflammatory [17], anticoagulative [18], cognition-enhancing [19], anti-hyperglycemic [20,21], neuroprotective [22], analgesic [23], antioxidative [24], immune regulatory [25,26], neuromuscular blocking [27], myelosuppression [13], and hepatic injury protective [28]. In 1998, the use of TGP as a rheumatoid arthritis (RA) modification drug was approved by the Chinese State Food and Drug Administration (SFDA). Similar to cancer growth, hyperplastic and invasive RA synovium expresses EGFR and its ligands [29]. Activation of EGFR signaling is responsible for synovial fibroblast proliferation in RA [29]. Furthermore, EGFR and its ligands can induce cytokine production of synovial fibroblasts during the pathogenesis of RA [29]. Agents that target EGFR have yielded promising results in animal experiments involving RA [29]. Since two most prominent members of EGFR family, EGFR and HER-2 are responsible for initiating multiple biological processes in vivo, the multiple biological functions of TGP intrigues us to hypothesize that both EGFR and HER-2 might be a target of TGP, and that TGP might work as a TKI in Lung Cancer Cell Lines.

Indeed, the cytotoxic effect of TGP seems to be the consequence of affecting several cellular processes. It has been reported that the albiflorin, a component of TGP, inhibits bladder cancer growth via phosphorylation of Chk2 [30]. Paeoniflorin, another component of TGP, induces cancer cell apoptosis via the Fas/Fas ligand (FasL) pathway [31]. Recently, it has also been demonstrated that paeoniflorin inhibits cell growth through cell cycle arrest specifically in G1 phase and causing apoptosis in human colon cancer HT29 cells [32].

We discovered in our studies that TGP inhibited both EGFR and HER-2 tyrosine phosphorylation in addition to suppressing EGFR and HER-2 expression in two lung cancer cell lines tested. At the same time, TGP showed anti-proliferation, anti-apoptosis, and cell cycle arrest activities. Based on current understanding of the signal transduction net work in cancer cells [33], all the desirable anti-lung cancer cell properties of TGP might be mediated through its ability to deactivate EGFR signaling by acting as a TKI and an EGFR expression suppressor. Therefore, TGP might be an interesting drug candidate for EGFR and HER-2 inhibition.

Materials and methods

Cell culture and TGP

Human lung cancer cell lines A549 and H1299 were obtained from the Chinese Academy of Sciences (Shanghai, China). They were maintained in RPMI 1640 media supplemented with 5% FBS, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin at 37°C with 5% CO₂. TGP was purchased in Liwah Pharmaceutical. TBS was used as the vehicle to deliver TGP [34].

Cell proliferation assay

A549 or H1299 (2000 cells/well) cells were seeded in 96-well plates. After 24 h incubation, the cells were treated with 200 μL complete media containing serial TGP. After 8, 32, and 56 h treatments, each well was added 20 μL of resazurin (2 mg/mL). The fluorescent signal was monitored using 544 nm excitation and 595 nm emission wavelengths by Spectramax M5 plate reader (Molecular Devices) [35]. The assay was performed in triplicate for each TGP treatment along with control. Same experiment was repeated twice.

NAC assay for cellular viability

A549 (2000 cells/well) cells were seeded in 96-well plates. After 24 h incubation, the cells were treated with 200 μL complete media containing serial TGP. After 8, 32, and 56 h treatments, each well was added 20 μL of resazurin (2 mg/mL). The fluorescent signal was monitored using 544 nm excitation and 595 nm emission wavelengths by Spectramax M5 plate reader (Molecular Devices) [35]. The absorbance was monitored at 570 nm using a Spectramax M5 plate reader (Molecular Devices).

Cell cycle analysis

A549 and H1299 cells (6×104 cells/well, 2 mL/well) were seeded in 6-well plates. After 24 h, the cells were treated with different concentrations of TGP in 2 mL of complete media. After another 24 h, media were collected and combined with adherent cells that were detached by brief trypsinization. Cell pellets were resuspended in 70% ethanol at 4°C overnight. After centrifugation, the supernatant was removed and cells were incubated with 0.5 mL PI/RNase Staining...
Buffer (BD Biosciences) for 15 min at room temperature. Cell cycle was analyzed using the Beckman cell analyzer FC500-mpl [36].

Detection of apoptosis

Apoptotic cells were quantified by Annexin V/propidium iodide (PI) double staining assay kit (BD Biosciences, San Diego CA, USA). Briefly, A549 and H1299 cells were treated with TGP the same as described for the cell cycle analysis. After washing with binding buffer, cells were suspended in 100 μl binding buffer containing Annexin V and PI, and incubated for 15 min at room temperature. The apoptotic cells were identified using the Beckman cell analyzer FC500-mpl.

Immunoblot analysis

Proteins from TGP treated A549 and H1299 cell lysates were quantified by BCA Protein Assay Kit [37]. Equal amount of proteins (50 μg) was resolved over 12% or 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membranes were blocked and then incubated with appropriate primary antibody overnight at 4°C. After incubation with appropriate secondary antibodies, the membranes were visualized using Western Lightning (PerkinElmer, USA).

Antibodies for Rb, CDK-2, CDK-4, CyclinD1, phospho-EGFR (Tyr1068), phospho-Akt (Ser473), HER-2, Phospho-HER2/ErbB2 (Tyr1248), Bax, Bcl-2, Caspase 3, PARP, EGFR, CyclinE1 and β-Actin were from Cell Signaling Technology (USA).

Results

TGP inhibits the growth of human lung cancer cells

The effects of TGP and Gefitinib were studied on the growth of A549 and H1299 human lung cancer cells. In both A549 (Figure 1B) and H1299 (Figure 1C) cells, proliferation of the cells was significantly suppressed in the presence of TGP in a concentration- and time-dependent manner compared with the positive control Gefitinib (Figure 1D). In A549 cells, TGP had IC50 values of 464.57, 124.78, 91.49 μg/mL in 24 h, 48 h, 72 h. Similar trend and even stronger effect was also observed in H1299 cells.

We used antioxidant NAC to eliminate possible influence of reactive oxygen species (ROS) for ROS has growth inhibition ability [38]. We optimized the concentrations of NAC that acts as a cellular ROS scavenger. Based on cell viability assay, the NAC concentration range used in A549 cells was 25-300 μM. At these concentrations, the lung cancer cell viability was not significantly affected by NAC. We used TGP at 80 μg/mL as positive controls in A549 cells to cause considerable growth inhibition (about 30%). However, the addition of NAC (from low to high concentrations) to TGP-treated cells did not affect the growth inhibition caused by TGP in A549 cells (Figure 1E). These results indicated that ROS was not associated with growth inhibition observed in Figure 1B.

TGP inhibits EGFR expression and phosphorylation

Data in Figure 1 showed that TGP inhibited the growth of two human lung cancer cells. Decades of research have shown that EGFR forms part of a complex signal transduction network that is essential for many important cellular processes, including cell proliferation, migration, survival and adhesion [39], we therefore tested if the observed growth inhibition was related to suppressed EGFR expression and/or phosphorylation of EGFR by TGP.

We examined the effect of TGP on the expression and activation of EGFR and the AKT. AKT is an important downstream effector of EGFR signaling. The phosphorylation of AKT (P-AKT) is conducted by phosphorylated EGFR (P-EGFR), followed by a survival signaling cascade.

As shown in Figure 2, TGP decreased the levels of EGFR, P-EGFR, P-AKT in a concentration-dependent manner in the two lung cancer cell lines. At 40 μg/mL, TGP decreased EGFR expression to 81% and phosphorylated EGFR to 58% compared to the treatment-free control in A549 cells (Figure 2A). Consistently, at 40 μg/mL, TGP decreased EGFR expression to 85% and phosphorylated EGFR to 46% compared to the treatment-free control in H1299 cells (Figure 2B). In both lung cancer cells, TGP reduced EGFR phosphorylation more than EGFR expression at all the concentrations tested, which indicated that TGP might act as a TKI.

TGP induces apoptosis in human lung cancer cells

Lapatinib, a clinically approved TKI, has been studied for its anti-cellular proliferation, pro-apoptosis, and cell cycle arrest properties in the same lung cancer cell line A549 [40] as used in our studies. Therefore, we decided to perform similar experiments with TGP but in both A549 and H1299 lung cancer cells.

The published report indicates that lapatinib induces apoptosis in A549 cells through analysis of key proteins in apoptotic pathway induced by lapatinib [40]. We determined the extent of apoptosis contributing to TGP treatment by flow cytometry analysis. To this end, A549 and H1299 cells were stained with Annexin V/propidium iodide and analyzed by flow cytometry. Viable cells (FITC-negative) and early apoptotic cells (FITC-positive) were PI-negative, whereas late apoptotic and necrotic cells were PI-positive and FITC-positive. In both A549 and H1299 cells, control cells showed only viable cells (K3) whereas treatment with TGP increased the percentage of early and late apoptosis. The results are shown in Figure 2B.
apoptotic cell population 27.7-fold compared to that of control cells in A549 cells. In A549 cells, treatment with TGP for 24 h increased cell signaling proteins TGP profoundly modified cell cycle and apoptosis-related progression and apoptotic pathway in lung cancer cells. Since TGP had the similar effects on both A549 and H1299 as data shown in Figures 1-3, we decided to use A549 cells for the following experiments. Western blotting was performed on the whole-cell lysates after the treatments with TGP. In the G1 checkpoint of the cell cycle, CyclinD1-CDK4/6 complex and CyclinE1-CDK2 complex are required to allow cell cycle progression through G1 phase. Our results showed that after 72h treatment, TGP decreased the levels of CDK-2, CDK-4, CyclinD1 and CyclinE1 in a concentration-dependent manner. Furthermore, Treatments with TGP increased the levels of total Rb (Figure 4A). Rb stands for retinoblastoma protein, which is a tumor suppressor protein that is dysfunctional in several major cancers. One function of Rb is to prevent excessive cell growth by inhibiting cell cycle progression.

Activation of HER-2 plays an important role in the process of apoptosis, since it has been reported that switching off HER-2 could lead to apoptosis [41]. In addition, the reported lapatinib [40] acts as a dual TKI for both EGFR and HER-2 in A549 cells. Thus we tested how TGP affects HER-2 expression and phosphorylation. Data in Figure 4B shows that TGP treatment decreased the expression of HER-2 and also the phosphorylation of HER-2 in a concentration-dependent manner. At 80 μg/ml, TGP decreased HER-2 expression to 64% and phosphorylated HER-2 to 21% compared to the treatment-free control in A549 cells. The data showed that TGP reduced HER-2 phosphorylation more than HER-2 expression at all the concentrations tested, which indicated that TGP might resemble lapatinib in that it acted as a dual TKI for both EGFR and HER-2.

Bcl-2, and Bax are members of the expanding Bcl-2 family that play key roles in the regulation of apoptosis [42]. Bcl-2 is an anti-apoptotic protein whereas Bax is a pro-apoptotic protein. Decreased ratio of Bcl-2/Bax allows Bax to act on mitochondrial membrane by releasing cytochrome c from mitochondria, which leads to cells apoptosis. It could be seen from Figure 4B that treatment of TGP decreased the Bcl-2 level in a concentration-dependent manner, coincided with no noticeable change in Bax. Consistent with decreased ratio of Bcl-2/Bax that induces apoptosis, caspase 3, a crucial player in the process of apoptosis, was significantly activated by the treatments with TGP in a concentration-dependent manner. PARP is a substrate of caspase 3. PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis [43]. Our data showed a concentration-dependent increase of the cleaved PARP as well (Figure 4B).

**Discussion**

EGF receptor family with their most prominent members EGFR and HER-2 represents validated targets for anti-cancer therapy. Anti-EGFR and HER-2 MoAbs (cetuximab, panitumumab, and trastuzumab) and TKIs (gefitinib, erlotinib, and lapatinib) have now been approved for the treatment of advanced colorectal cancer, squamous cell carcinoma of the head and neck, advanced NSCLC, as well as pancreatic and breast cancer. However, the benefits of costly TKIs and Anti-EGFRs MoAbs to most cancer patients are very limited due to complications of drug-resistant and side effects. The median survival rates increase only for a few months compared to patients treated with conventional therapies.

Our data showed that TGP inhibited both EGFR and HER-2 tyrosine phosphorylation in addition to suppressed EGFR and HER-2 expression in two lung cancer cell lines tested. Subsequently, TGP showed expected anti-proliferation, anti-apoptosis, and cell cycle arresting properties of EGFR/HER-2 TKI in the cancer cell lines tested.
Therefore, TGP is promising in treating EGFR/HER-2 over-activated cancers, including NSCLC, breast, squamous cell carcinoma of the head and neck and others. Based on current understanding of the signal transduction net work in cancer cells [33], the desirable anti-lung cancer cell properties of TGP might be mediated through its ability to deactivate both EGFR and HER-2 signaling pathways by acting as a TKI and an EGFR and HER-2 expression suppressors. Hence our studies of TGP might provide with useful information towards developing inexpensive and more effective anti-EGFR drugs for cancer patients.

There is considerable interest in finding anticancer drugs among Chinese medicinal herbs. Currently, the cost of developing an approvable drug is frequently cited as about $1 billion across 15 years in the US, although recent estimates have ranged as high as $4 billion to $12 billion per drug, depending upon how many failures are included in the estimate. Therefore, it will be a daunting job to develop novel and cheaper TKIs. In a recently published study, it was discovered that among 656 US FDA approved drugs tested, each drug hits more than 7 targets in the 73 total targets tested [44]. Many drugs are less effective to the previously known targets compared to off-targets based on the affinity or biological data. This published report provides valuable guidance in efforts to repurpose existing drugs, especially thousands of established herb-based medicines, for new diseases and conditions. Our studies of TGP are a good proof of this ability to deactivate both EGFR and HER-2 signaling pathways by acting as a TKI and an EGFR and HER-2 expression suppressors. Therefore, our studies of TGP might provide with useful information towards developing inexpensive and more effective anti-EGFR drugs for cancer patients.

![Figure 4: Effects of TGP on cell cycle and apoptosis related proteins in A549 cells. A549 cells were seeded in 10-cm dishes for 24 h, then cells were treated with serial concentrations of TGP. After 72 h of incubation, cells were harvested for immunoblot analysis as described in Methods. The numbers underneath of the blots represent band intensity (normalized to β-Actin, means of three independent experiments) measured by Image J software. The standard deviations (all within ± 15% of the means) were not shown. β-Actin was served as an equal loading control. The experiments were repeated three times.](image)

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